

Molecular phylogeography of *Boophis tephraeomystax*: a test case for east-west vicariance in Malagasy anurans

(Amphibia, Anura, Mantellidae)

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Phylogenetic analysis of sequences of the mitochondrial 16S rRNA gene in the Malagasy treefrog *Boophis tephraeomystax* resulted in a clear separation of one clade corresponding to the populations from the east and northwest and a second clade comprising the populations from the west of Madagascar. Within each clade, the pairwise differentiation was 0-3 nucleotides, whereas differentiation between clades was 12-13 nucleotides. This phylogeographic structure correlates with bioacoustic and karyological differences between eastern and western populations. We consider the observed differences as indicative of separation on the species level, and revalidate *Rhacophorus doulioti* Angel, 1934 as *Boophis doulioti* to refer to the western populations hitherto assigned to *B. tephraeomystax*. *B. tephraeomystax* and *B. doulioti* are an additional example of a pair of sister species distributed allopatrically in eastern and western Madagascar. This pattern may have been caused by vicariant speciation in the course of past climatic shifts which led to more or less temporary isolation and fragmentation of western amphibian populations.

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Introduction

Species distribution patterns in the amphibian fauna of Madagascar are highly influenced by the climatic diversity of the island (Lees 1996). Eastern Madagascar receives high amounts of rainfall, while the west and especially the south-west are seasonal dry to arid regions. This difference is largely caused by the central mountain chain, spanning from north to south, which acts as a barrier for the humidity carried by trade winds from the east (Battistini 1996).

The highest amphibian diversity in Madagascar is found in the eastern rainforests (Lees 1996), but recent studies (Glaw & Vences 1997, Glaw et al. 1998, Vences et al. 1999, 2000a) indicated that western Madagascar is also an important center of diversity and, especially, endemism for amphibians. However, a few species of amphibians are currently known from both low elevations along the eastern as well as the western coast. In reptiles, recent discoveries indicated that eastern and western populations of several forms are distinct at the species

level and possibly are witnesses of climatic shifts in the past, when more humid conditions prevailed also on Madagascar's west coast (Nussbaum & Raxworthy 1998, Nussbaum et al. 1998).

The treefrog *Boophis tephraeomystax* is a widespread generalist species occurring in low- to mid-elevational regions throughout Madagascar (Blommers-Schlösser & Blanc 1991; Glaw & Vences 1994). As the species apparently is unable to colonize highland regions, it is likely that the central mountain chain acts as a barrier, inhibiting gene flow between eastern and western populations. Contact zones exist, however, around Tolagnaro in southern Madagascar, and in the Sambirano region in the northwest.

Previous studies on intraspecific variation of *Boophis tephraeomystax* are limited, but Blommers-Schlösser (1978) reported on important karyological differences between species from eastern (Toamasina and Foulpointe) and southwestern Madagascar (Toliara).

In the present study, we analysed mitochondrial DNA sequences from seven *Boophis tephraeomystax* populations throughout its range and compared advertisement call data from a total of 11 populations. Our aim was to find evidence for phylogeographic structuring (Avice 2000) of the haplotypes found within the species, and to discuss the observed pattern in the light of possible vicariant east-west differentiation of Malagasy anurans.

Materials and Methods

We sequenced fragments of the mitochondrial 16S rRNA gene of 562 nucleotides (nt) using primers and protocols given in Vences et al. (2000b). Vouchers were deposited in the herpetological collections of the Université d'Antananarivo, Département de Biologie Animale (UADBA), the Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn (ZFMK) and the Zoologische Staatssammlung München (ZSM). Specimens of *Boophis tephraeomystax* used (Genbank/EMBL accession of sequences and voucher specimen numbers in parentheses) were from (1) Nosy Be (ZSM 458/2000, AJ312114), (2) Ambanja (ZSM 489/2000, AJ312115), (3) Sambava (UADBA (FG/MV) 2000. 379, AJ312116), (4) Cap Est, Masoala Peninsula (AF215333; ZFMK 66685), (5) Ampijoroa, Ankarafantsika Reserve ZSM 704/2001, AJ312117), (6) Kirindy (AF215334; ZFMK 66690), and (7) Isalo (AF 215332; ZFMK 70495). We used a specimen of *Boophis xerophilus* from Kirindy (ZFMK 66705, AF 215335), which is a close relative

of *B. tephraeomystax*, as outgroup.

Sequences were aligned using SEQUENCE NAVIGATOR (Applied Biosystems). Inclusion of gaps to account for indels was only necessary at a single position. The aligned sequences were submitted to analysis using PAUP*, version 4.0 (Swofford 1998). We performed Maximum Parsimony analyses using the exhaustive search option and Neighbor joining analyses using LogDet distances which are robust against possible variation of sequence evolution among lineages (Lockhart et al. 1994). Homogeneity of base frequencies across sequences was tested using algorithms implemented in PAUP*.

Advertisement calls were recorded in the field using different portable tape recorders and microphones. Detailed call descriptions will be published elsewhere; we here provide only short informations on the main differences that are obvious to the human ear.

Results and discussion

A chi-square test did not contradict homogeneity of base frequencies across taxa ($df = 21; P = 1$). Of the 562 characters included in the analysis, 516 were constant, 33 variable but parsimony-uninformative, and 13 parsimony-informative. Maximum parsimony analysis resulted in two equally parsimonious trees (51 steps; consistency index 0.98, retention index 0.96). The resulting strict consensus tree (Fig. 1) divided the *B. tephraeomystax* sequences into two clearly distinct clades, corresponding to the eastern and Sambirano (Cap Est, Sambava, Ambanja, Nosy Be) and western (Ankarafantsika, Kirindy, Isalo) populations. Within the first clade, the Ambanja sequence formed a subclade with the Nosy Be sequence. The Neighbor-joining analysis resulted in a similar topology. Bootstrap analyses (Felsenstein 1985) with 2000 replicates resulted in high support ($> 95\%$) for these clades using either method.

Genetic differentiation was 0-3 nt among sequences in the eastern/Sambirano clade (the sequences from Nosy Be and Ambanja were identical), and 1-2 nt among sequences in the western clade, but 12-13 nt (2% pairwise sequence divergence) between clades.

The genetic data therefore clearly indicate the presence of different haplotypes in eastern and western populations of *Boophis tephraeomystax* (Fig. 2). In our extensive data set on Malagasy anurans, we never observed intrap-

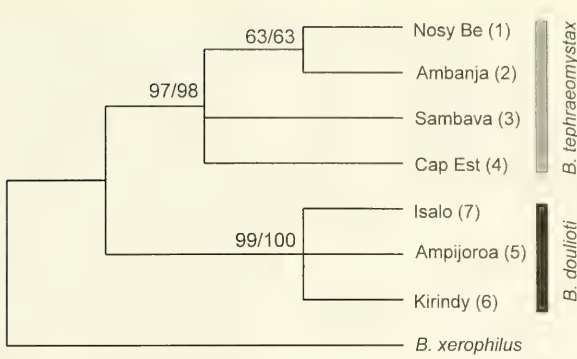


Fig. 1. Strict consensus of two equally most parsimonious trees obtained by Maximum Parsimony analysis of sequences (562 bp of the 16S rRNA gene) from seven populations previously assigned to *Boophis tephraeomystax*. Numbers above branches indicate bootstrap support (Felsenstein 1985) of Maximum Parsimony and Neighbor-joining analyses, respectively. *Boophis xerophilus* was used as the outgroup.

opulational polymorphism of such magnitude in the 16S gene; although we here sequenced only a single specimen per population, it can safely be excluded that the observed differences between populations are also present within populations. The differentiation of eastern and western populations of *B. tephraeomystax* is furthermore corroborated by bioacoustic and karyological differences.

The advertisement call of *B. tephraeomystax* was first described by Blommers-Schlösser (1979), who characterized it as reminding the yelping of a young dog. In a previous publication (Glaw & Vences 1994) we confirmed this characterization, but mentioned that these vocalization can be emitted as single notes or as short note series, or can be followed by a short click. We also noted that highly motivated males from Ranohira (near Isalo) had a more complex call structure, with a faster note repetition rate and including additional trill notes of 5-8 pulses. During intensive survey work in 1995-1996 and 2000-2001, we heard choruses of *Boophis tephraeomystax* at many additional sites, and found that the observed differences were largely not correlated with the male's state of sexual motivation. At eastern sites (including the Sambirana Region), the calls were generally single yelping notes repeated after longer intervals. This was true in Tolagnaro, Ranomafana, Andasibe, Vohidrazana, Maroantsetra, Sambava, Antsirasira, Ambanja, and Nosy Be. In several cases (e.g., on the northwestern offshore island Nosy Be), the specimens were calling in a highly motivated chorus. Two females were ob-

served at Nosy Be approaching the breeding pond and mating with two of the calling males, but nevertheless no trill calls were heard in this population. On the other hand, at Tolagnaro, Ranohira, Kirindy and Ankarafantsika, trill calls and note series were usually part of the calling repertoire. At Ankarafantsika, after a few days of drought, specimens were very shy and little motivated; nevertheless, trill calls were regularly heard, as in highly motivated choruses after heavy rain at this site (though less frequently). The sonagram of a call recorded at Toliara in Blommers-Schlösser (1979) also shows two rapidly repeated yelping notes followed by a short trill of three pulses. We therefore conclude that the observed differences are geographically correlated patterns: western populations of *B. tephraeomystax* have complex calls with trill notes, while eastern populations usually emit only single yelping notes.

The karyological differences reported by Blommers-Schlösser (1978) between southwestern and eastern populations of *B. tephraeomystax* were significant. All specimens had a karyotype of $2n = 26$. However, in eastern specimens, the chromosome pairs 2, 4 and 6 were submetacentric (the remaining pairs being metacentric), while this state was expressed in pairs 2, 3, 4, 9 and 10 in the southwestern specimens. A secondary constriction was noted on pair 6 in the eastern specimens and on pair 10 in the southwestern specimens. These differences led Blommers-Schlösser & Blanc (1991) to pose the question whether these two populations may correspond to different species.

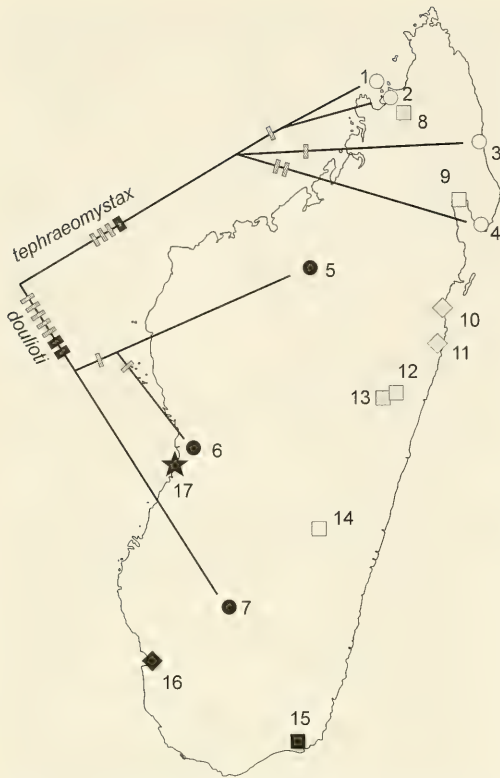


Fig. 2. Localities of populations that can be assigned to *Boophis tephraeomystax* (grey symbols) and *Boophis doulioti* (black symbols). Circles refer to populations from which DNA sequences were included in the present study; squares refer to populations of which call data are available (additionally also from all populations symbolized by circles except for Cap Est, and from Toliara); rhomboids mark sites which were sampled for karyological data by Blommers-Schlösser (1978); and the star marks the type locality of *Boophis doulioti*. Localities 1-7 as in Fig. 1 and in Materials and Methods: 1. Nosy Be. 2. Ambanja. 3. Sambava. 4. Cap Est. 5. Ampijoroa. 6. Kirindy. 7. Isalo. Other localities: 8. Antsiraisira. 9. Maroantsetra. 10. Foulpointe. 11. Toamasina (= Tamatave). 12. Vohidrazana. 13. Andasibe. 14. Ranomafana. 15. Tolagnaro (= Fort Dauphin). 16. Toliara (= Tulear). 17. Morondava. The cladogram (one of the two most parsimonious trees from Fig. 1; 51 steps, consistency index 0.95) is superimposed on the map; branch lengths were adjusted to fit the geography and are not indicative of genetic distances. Bars mark apomorphies in sequence evolution as reconstructed using PAUP* (Swofford 1998); thin grey bars are transitions, thick black bars are transversions.

Evaluating the differences summarized above, there remains little doubt that this question can be answered positively. The amount of karyological, bioacoustic and genetic differentiation indicates that *B. tephraeomystax* actually is a complex of two allopatrically distributed species. We were unable to detect reliable differences between these two species in external morphology; a detailed morphometric study including large sample sizes is necessary to address this question.

B. tephraeomystax was described from Madagascar, without precise locality; specimens from Nosy Be were later erroneously also considered to be part of the type series (Glaw & Vences 1997). Despite the uncertain type locality, it can be assumed that the type was collected from eastern Madagascar or the Sambirano region, as most early anuran collections from Madagascar were made in these areas. We therefore consider the form occurring in these areas as *B. tephraeomystax*. Origin of the types of the junior synonyms *Polypedates crossleyi* Peters, 1874 (Nosy Vola) and *Polypedates dispar* Boettger, 1879 (Nosy Be) are also in the eastern respectively Sambirano regions (see Blommers-Schlösser & Blanc 1991). *Rhacophorus hildebrandti* Ahl, 1925 was described from "north-western Madagascar"; according to Beentje (1998), the collector J. M. Hildebrandt spent only little time during the dry season in the Ankarafantsika region, while he collected during more extended periods in the rainy season in the Sambirano Region. It is unlikely that the type of *hildebrandti* was collected in the seasonal Ankarafantsika environments during the dry season, and we therefore suppose that it originates from the Sambirano Region (and is therefore a synonym of *B. tephraeomystax*). The only junior synonym from western Madagascar is *Rhacophorus doulioti* Angel, 1934. This taxon was described from Morondava, which is close to our sampling locality Kirindy. We therefore propose to resurrect this name as *Boophis doulioti* (Angel, 1934) from the synonymy of *Boophis tephraeomystax*, and to apply it to the populations in western Madagascar between Ankarafantsika and Fort Dauphin.

The partition of *Boophis tephraeomystax* into two separate species, *B. tephraeomystax* and *B. doulioti*, adds relevant perspective to the biogeography of Madagascar. Only few anurans

are known to occur in eastern as well as in western Madagascar (Blommers-Schlösser & Blanc 1991; Glaw & Vences 1994; Hawkins 1994; Emanuelli & Jesu 1995; Vences et al. 1999): *Ptychadena mascareniensis* is ubiquitous in Madagascar, including east and west; *Mantella betsileo* and *Mantidactylus wittei* (subgenus *Blommersia*) show a coastal distribution which is rather similar to that of *B. tephraomystax*; *Scaphiophryne marmorata* occurs at the western site Tsingy de Bemaraha and in eastern rainforests; *Boophis luteus* is known from the east and from Isalo; *Mantidactylus ulcerosus*, *M. biporus*, *M. curtus* and *M. betsileanus* (all subgenus *Brygoomantis*) have been recorded from several eastern, north-western and western sites; *Mantidactylus luteus* (subgenus *Gephyromantis*) and *M. granulatus* (subgenus *Phylacomantis*) are known from the east and from one western site, respectively.

Of these examples, several are probably due to wrong determinations, such as the records of the different *Brygoomantis* which are very difficult to distinguish morphologically; so far, all *Brygoomantis* found by us in western Madagascar were assignable to *M. ulcerosus* or to undescribed species. The record of *Mantidactylus luteus* (Blommers-Schlösser & Blanc 1991, apparently based on MNHN 1975.327) is due to a mistake with *M. corvus* or *M. pseudoasper* (pers. obs.). The record of *M. granulatus* from the western locality Antsingy by Blommers-Schlösser & Blanc (1991) is unconfirmed as we were unable to locate vouchers for this locality in the MNHN and ZMA collections on which the distribution maps of these authors were almost entirely based. The *Scaphiophryne marmorata* population from the Tsingy de Bemaraha shows different colour patterns to those from the east, and may correspond to a distinct species. And at least in *Mantella betsileo*, genetic evidence (pers. obs.) indicates that the western populations are taxonomically distinct from those in the east and northwest. No data have so far been published on the western populations of *Mantidactylus wittei* and *Boophis luteus*, but further studies may confirm that the anuran endemismity rates of western Madagascar are much higher than previously thought.

On the other hand, at least four pairs of amphibian sister species show a vicariant east-west distribution: *Boophis albilabris* (east) and *B. occidentalis* (west), *Mantidactylus pseudoasper*

(northeast and northwest) and *M. corvus* (west), *Heterixalus betsileo* (east) and *H. carbonei* (west), *Aglyptodactylus madagascariensis* (east) and *A. securifer* (west) (Glaw & Vences 1994, Glaw et al. 1998, Vences et al. 2000a). The present study adds *Boophis tephraomystax* (east and northwest) and *B. doulioti* (west) as a further example to this list.

A scenario which could explain this pattern assumes the existence of a more humid climate which allowed the respective ancestors of these species pairs to populate continuously eastern and western Madagascar. In a second step, more arid climate arose and isolated the western populations during a considerable amount of time in small humid refuges. During this time of isolation, allopatric speciation of the vicariant populations occurred. Subsequent shifts back to more humid conditions allowed a secondary contact between some species pairs. To test this hypothesis, it will be necessary to study more variable genetic markers and to identify in detail the contact and/or hybrid zones of the species pairs.

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Appendix

The taxon *Rhacophorus doulioti*, as described by Angel (1934), is based on two specimens housed in the Muséum National d'Histoire Naturelle, Paris (MNHN): the lectotype (MNHN 1891.356) and the paralectotype (MNHN 1891.357) (Glaw & Vences 1997). Measurements of the lectotype (paralectotype in parentheses), taken with a caliper to the nearest 0.1 mm, are: snout-vent length 37.2 (31.3); head width 12.5 (11.5); head length 14.1 (12.3); horizontal eye diameter 3.6 (3.5); eye-nostril distance 3.1 (2.8); nostril-snout tip distance 2.4 (2.1); nostril-nostril distance 3.4 (3.1); horizontal tympanum diameter 2.6 (2.3); hand length 10.8 (8.8); forelimb length 20.6 (17.1); hindlimb length 56.4 (50.5); foot length 15.4 (13.8); foot length including tarsus 24.3 (22.0); length of inner metatarsal tubercle 1.4 (1.4). When the hindlimb is adpressed along the body, the tibiotarsal articulation reaches between eye and nostril in both specimens. Webbing formula (according to Blommers-Schlösser & Blanc 1991) is 1(0), 2i(1), 2e(0), 3i(1), 3e(0), 4i(1.25), 4e(1), 5(0) in the lectotype and 1(0.25), 2i(1.25), 2e(0), 3i(1.5), 3e(0), 4i(1.5), 4e(1.25), 5(0.25) in the paralectotype.